VIROLOGY 215, 31–39 (1996)
Article No. 0004

Biochemical and Genetic Evidence for the Intermolecular Association of the RNA-Dependent Protein Kinase PKR from Human Cells

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Received August 11, 1995; accepted October 19, 1995

The interferon-inducible protein kinase (PKR) is activated by an RNA-dependent autophosphorylation. Structure–function studies of the 551 amino acid PKR kinase from human cells have revealed that catalytic-deficient PKR mutants such as PKR(1–551)K296R display a dominant negative behavior when expressed in transfected cells. The potential for PKR to form protein multimers has therefore been examined. Three types of studies, including both genetic and biochemical analyses, demonstrated that PKR from human cells undergoes an intermolecular association that is not dependent upon RNA. First, the intermolecular association of PKR *in vitro* was demonstrated in the context of an enzyme–substrate interaction. Purified recombinant histidine-tagged PKR(1–551)K296R mutant protein was phosphorylated by purified wild-type PKR; this intermolecular phosphorylation of PKR was dependent on double-stranded RNA. At a fixed RNA concentration, high concentrations of the HIS-PKR(1–551)K296R mutant both impaired the autophosphorylation of wild-type PKR and blocked the trans-phosphorylation of itself. Second, the yeast two-hybrid system was used to probe the intermolecular association of PKR *in vivo.* Coexpression of the full-length catalytic-deficient phosphotransfer mutant PKR(1–551)K296R as a fusion protein with the Gal4 activation domain and the Gal4 DNA binding domain resulted in the expression of two Gal4-responsive reporter genes, *HIS*3 and *lac*Z. The full-length RNA-binding deficient PKR(1–551)K64E/K296R double mutant also interacted with PKR(1–551)K296R sufficiently to activate Gal4-responsive reporter genes; however, other PKR mutants including PKR(1–280)wt and PKR(281–551)K296R as well as p53, RAS, and BCL2 did not. Third, both PKR(1–551)K296R and PKR(1–551)K64E/K296R enhanced the expression of the reovirus S1 gene and S1/S4 chimeric gene in cotransfected COS cells. By contrast, the expression of the reovirus S4 gene was not enhanced by cotransfection with either PKR(1–551)K296R or PKR(1–551)K64E/K296R. These results indicate that PKR interacts with itself in an intermolecular manner both *in vivo* and *in vitro,* and that RNA binding is neither necessary nor sufficient for PKR multimerization.

INTRODUCTION

The RNA-dependent protein kinase (PKR)[5] is an important regulator of translation in interferon-treated and virus-infected animal cells (Samuel, 1991; Schneider and Shenk, 1987). PKR acquires protein serine/threonine kinase activity following autophosphorylation, a process mediated by RNA with double-stranded character (Samuel, 1993; Mathews, 1993). The best characterized of the PKR substrates is protein synthesis initiation factor eIF-2. Following activation, PKR catalyzes the phosphorylation of eIF-2 on serine 51 of the α subunit (Samuel, 1979; Pathak *et al.,* 1988). Serine-51 phosphorylation of eIF-2α leads to an inhibition of translation (Hershey, 1989; Samuel, 1993). PKR can also catalyze the phosphorylation of transcription factor inhibitor I-κB, which leads to activation and nuclear translocation of the transcription factor NF-κB (Kumar *et al.,* 1994). The amount of active PKR kinase present within cells is regulated at multiple levels: at the transcriptional level by IFN treatment (Meurs *et al.,* 1990; Tanaka and Samuel, 1994); at the translational level by an autoregulatory mechanism (Thomis and Samuel, 1992; Barber *et al.,* 1993); and at the posttranslational level by the RNA-mediated autophosphorylation of PKR (Samuel, 1979; Krust *et al.,* 1984; Berry *et al.,* 1985).

PKR is an important component of the IFN-induced antiviral response (Samuel, 1991). Two of the most compelling lines of evidence for the involvement of PKR in the antiviral action of IFN were provided from the analysis of virus-derived inhibitors of PKR function, such as adenovirus VA_I RNA (Mathews and Shenk, 1991), and from

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[5] Abbreviations used: PKR, the RNA-dependent protein kinase, also known as the P1/eIF-2α protein kinase, DAI, P1, and p68; eIF-2α, the α subunit of protein synthesis initiation factor eIF-2; IFN, interferon; dsRNA, double-stranded RNA; bp, base-pair; nt, nucleotide.

0042-6822/96 $12.00

the analysis of cells transfected with PKR cDNA clones (Meurs *et al.,* 1992; Lee and Esteban, 1993). Expression in mouse NIH 3T3 cells of the cDNA encoding wild-type human PKR mediates the phosphorylation of eIF-2α and, more importantly, reduces the replication of encephalomyocarditis virus (Meurs *et al.,* 1992) and vaccinia virus (Lee and Esteban, 1993). By contrast, transfection with a mutant cDNA encoding PKR which lacks kinase activity, for example the catalytic subdomain II point mutant PKR(1–551)K296R, did not reduce virus yields (Meurs *et al.,* 1992; Lee and Esteban, 1993).

In addition to effects on viral gene expression, PKR also is implicated in the control of cell proliferation (Lengyel, 1993). Expression of functionally defective human PKR cDNA in mouse 3T3 cells causes malignant transformation. Furthermore, stable transformants of 3T3 cells overexpressing catalytically inactive human PKR protein, such as PKR(K296R), are highly tumorigenic when injected into nude mice (Koromilas *et al.,* 1992; Meurs *et al.,* 1993). Consistent with the possible tumor suppressor function and role of PKR in the control of cell proliferation (Lengyel, 1993) is the observation that human PKR(wt) but not PKR mutants lacking catalytic activity mediate a growth suppression response in yeast (Chong *et al.,* 1992; Romano *et al.,* 1995).

Catalytically inactive forms of PKR, exemplified by PKR(1–551)K296R and the truncated PKR(1–243), display dominant negative effects on the function of wild-type PKR. For example, inactive forms of PKR can enhance the synthesis of reporter proteins in cotransfected animal cells (Barber *et al.,* 1993; Henry *et al.,* 1994), can rescue rabbit reticulocyte protein synthesis activity *in vitro* from the inhibitory effects of dsRNA (Sharp *et al.,* 1993), and can impair the phosphorylation of eIF-2α (Dever *et al.,* 1993) and reverse the growth suppression phenotype (Chong *et al.,* 1992; Romano *et al.,* 1995) mediated by PKR(1–551)wt in yeast. The mechanism of the dominant negative effects of inactive PKR proteins has not yet been resolved. As an extension of our studies of the structure and function of the PKR kinase, we have used both genetic and biochemical approaches to examine the potential for PKR to form protein multimers. Our results are consistent with the RNA-independent intermolecular association of one PKR molecule with a second PKR molecule, both *in vitro* and *in vivo.*

MATERIALS AND METHODS

Materials

Wild-type and mutant forms of the human PKR cDNA were previously described as follows: wild-type wt(1–551) and the catalytic-negative subdomain II mutant K296R(1–551) (Thomis and Samuel, 1992); the double mutant K64E/K296R(1–551), which is RNA-binding negative (McCormack *et al.,* 1994); and the N-terminal histidine-tagged K296R mutant His-K296R(1–551) (Thomis and Samuel, 1993). The reovirus S1(wt), S4(wt), and S1/S4 chimeric gene expression vector constructions were as previously described (Munemitsu and Samuel, 1988; Henry *et al.,* 1994). The yeast Gal4 DNA binding (pAS1-CYH2) and activation (pACTII) domain plasmid vectors were generously provided by Dr. S. J. Elledge (Baylor College of Medicine, Houston) (Durfee *et al.,* 1993). DNA binding domain plasmids (pGBT9 and pGBT10) were as described in Van Aelst *et al.* (1993). Rabbit polyclonal antiserum prepared against recombinant TrpE–PKR fusion protein was as previously described (Thomis *et al.,* 1992). PKR(wt) kinase was purified from ribosomal salt-washes from IFN-treated human amnion U cells as previously described (Berry *et al.,* 1985), except that the procedure was modified to include a MonoQ ion-exchange fast protein liquid chromatography (FPLC) step. His-PKR(K296R) protein was purified from the 100,000 *g* supernatant fraction of recombinant baculovirus-infected Sf21 cells by Ni^{2+}-chelation Sepharose column chromatography and followed by MonoS FPLC ion-exchange chromatography as previously described (Thomis and Samuel, 1993).

PKR interaction assays in the yeast two-hybrid system

PKR–PKR homomeric interaction *in vivo* in yeast was measured by activation of *HIS*3 and *lac*Z reporter constructs as detected by growth and color assays, respectively (Durfee *et al.,* 1993). The yeast strain Y190 [*MAT*a-*leu2-3, 112, ura3-52, trp1-901, his3-*Δ*200, ade2-101, gal4* Δ*gal80* Δ + *URA3*::*GAL* → *lacZ, LYS2*::*GAL* → *HIS3, cyh*^{r}] was the transformation recipient for all plasmid constructions. The indicated GAL4–PKR fusions in the yeast plasmid vectors pAS and pACT were constructed following standard procedures for DNA manipulations (Sambrook *et al.,* 1989; Rose, 1990). Y190 was transformed to either tryptophan or leucine prototrophy with pAS-PKR(K296R) or pACT-PKR(K296R), respectively. A single colony was then grown in YPD (yeast-peptone-glucose) media and transformed with the corresponding test plasmid using salmon sperm DNA as a carrier. The transformation mixture was plated on 15-cm petri dishes containing SC (synthetic complete) media lacking L-leucine and L-tryptophan; incubation was at 30° for 2–3 days. Single colonies were then tested for growth at 30° on SC media that lacked L-leucine, L-tryptophan, and L-histidine but contained 25 m*M* 3-aminotriazole (Sigma). β-galactosidase activity was determined both by the colony nitrocellulose lift method and in extracts prepared from liquid cultures.

For the filter lift assay for β-galactosidase activity (Breeden and Nasmyth, 1985), nitrocellulose filters from the SC-leu$^-$trp$^-$ plates were used. The filters were permeabilized by freezing in liquid nitrogen (10 sec) and thawed at room temperature, overlaid on Whatman 3MM paper saturated with Z-buffer containing 1 mg/ml X-gal, and then incubated at 30° for color development. For the

liquid culture assay for β-galactosidase activity (Rose, 1990), cultures (5 ml) were grown in YPD to an OD_{600} of 1.0–1.2. Cells were harvested, disrupted by vigorously vortexing (10 times at high speed for 15 sec each, with cooling on ice between bursts) in 0.1 *M* Tris–Cl buffer, pH 8.0, containing glass beads (400–650 μm) and 1 m*M* dithiothreitol, 20% v/v glycerol, and 1.8 m*M* PMSF. The cell-free extract was used to determine β-galactosidase activity using *O*-nitrophenyl-β-D-galactopyranoside (ONPG) as described (Rose, 1990).

Assays for PKR

[γ-^{32}P]ATP-mediated autophosphorylation of PKR was measured as previously described (Samuel *et al.,* 1986; Thomis and Samuel, 1993). Western immunoblot analysis of PKR was as previously described using rabbit polyclonal antiserum against PKR at a dilution of 1:750 and ^{125}I-labeled protein A to detect antibody–antigen complexes (Thomis *et al.,* 1992).

Cell maintenance, transfection, and measurement of protein synthesis *in vivo*

Monkey kidney COS cells were grown in monolayer culture and transfection was by the DEAE-dextran/chloroquine phosphate method as previously described (Luthman and Magnusson, 1983; Munemitsu and Samuel, 1988). Cotransfections were performed using 5 μg of the reovirus S gene expression construction or pJC119 vector alone and 5 μg of the PKR expression construction. The synthesis of reovirus proteins was measured at 48 hr after transfection by pulse-labeling with [^{35}S]-methionine for 60 min, preparation of extracts by Nonidet P-40 lysis, immunoprecipitation with a saturating amount of rabbit polyclonal antibody and formalin-fixed *Staphylococcus aureus,* and analysis of the proteins by SDS–polyacrylamide gel electrophoresis and autoradiography. Protein synthesis was quantified by scanning autoradiograms with an LKB Ultroscan XL laser densitometer. The methods have previously been described in detail (Samuel and Brody, 1990; Henry *et al.,* 1994).

RESULTS

Mutant forms of the RNA-dependent protein kinase PKR often display a dominant negative property in transfected cells. For example, the synthesis of reporter proteins is enhanced and cell growth suppression is reversed by expression of PKR proteins lacking catalytic activity (Barber *et al.,* 1993, 1995; Chong *et al.,* 1992; Henry *et al.,* 1994; Koromilas *et al.,* 1992; Meurs *et al.,* 1993; and Romano *et al.,* 1995). The mechanism by which PKR mutants act as dominant negative inhibitors of PKR function remains unresolved, but may involve the formation of inactive heterodimers of PKR. We have utilized biochemical and genetic approaches to test the ability of PKR to undergo intermolecular association with itself.

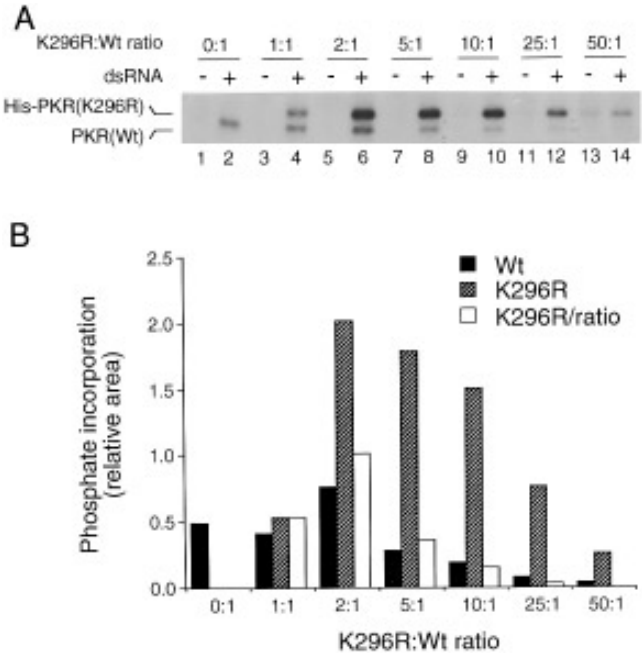

FIG. 1. Effect of His-PKR(K296R) protein concentration on the phosphorylation catalyzed by PKR(wt). (A) *In vitro* phosphorylation reaction mixtures contained either no added dsRNA (−) or 0.1 μg/ml of poly[rI]:poly[rC] (+) as indicated. Lanes 1 and 2 (0:1) contained only PKR(wt). Lanes 3 to 14 contained increasing amounts of His-PKR(K296R) as follows: lanes 3 and 4 (1:1) contained equimolar amounts of PKR(wt) and His-PKR(K296R); lanes 5 and 6 (2:1) contained a 2-fold, lanes 7 and 8 (5:1) contained a 5-fold, lanes 9 and 10 (10:1) contained a 10-fold, lanes 11 and 12 (25:1) contained a 25-fold, and lanes 13 and 14 (50:1) contained a 50-fold molar excess, respectively, of His-PKR(K296R) relative to PKR(wt). Reaction mixtures were incubated at 30° for 2 min, and ^{32}P-labeled phosphoproteins were fractionated by SDS–PAGE (10% polyacrylamide gel) and visualized by autoradiography. The positions of PKR(wt) and His-PKR(K296R) phosphoproteins are indicated. (B) Quantitation of the autoradiogram shown in A by laser densitometry. Closed bars represent PKR(wt), hatched bars represent His-PKR(K296R), and open bars represent the relative amount of phosphate incorporated into His-PKR(K296R), which was calculated by dividing the total amount of phosphate incorporated into His-PKR(K296R) by the K296R:wt ratio.

Intermolecular autophosphorylation of PKR is impaired by excess His-PKR(K296R)

The phosphorylations of purified His-PKR(K296R) and PKR(wt) were examined in reaction mixtures containing a constant amount of PKR(wt) and increasing amounts of His-PKR(K296R). *In vitro* phosphorylated ^{32}P-labeled products were analyzed by SDS–PAGE and autoradiography (Fig. 1A); phosphate incorporation was quantified by laser densitometry of the autoradiogram (Fig. 1B). His-PKR(K296R) contains an engineered N-terminal polyhistidine tag that adds about 4 kDa to the size of PKR(K296R), a mutant of PKR that lacks catalytic activity (Katze *et al.,* 1991; Thomis and Samuel, 1992). The His-PKR(K296R) and PKR(wt) proteins can be separated clearly from each other by gel electrophoresis on SDS–PAGE gels (Thomis and Samuel, 1993). This is demonstrated in Fig. 1A; His-PKR(K296R) and PKR(wt) were de-

tected as two distinct bands of 71 and 67 kDa, respectively (lane 4).

Both the autophosphorylation of PKR(wt) and the trans-phosphorylation of His-PKR(K296R) were dependent on the presence of poly[rI]:poly[rC] dsRNA (Fig. 1A, even-numbered lanes). No phosphorylation of either PKR(wt) or His-PKR(K296R) was observed in the absence of activator dsRNA (Fig. 1A, odd-numbered lanes). However, in the presence of poly[rI]:poly[rC] RNA, two phosphoproteins were detected, autophosphorylated PKR(wt) (lanes 2 and 4, for example) and trans-phosphorylated His-PKR(K296R) (lane 4, for example). When His-PKR(K296R) and PKR(wt) were present at equimolar concentration (Fig. 1A, lane 4, and Fig. 1B), the amount of phosphorylation of the two PKR proteins was comparable. Phosphorylation of His-PKR(K296R) was maximal when present at a twofold molar excess over PKR(wt) (Fig. 1A, lane 6, and Fig. 1B). Addition of His-PKR(K296R) in amounts greater than a twofold molar excess over that of PKR(wt) resulted in a concentration-dependent decrease of both His-PKR(K296R) trans-phosphorylation and PKR(wt) autophosphorylation (Fig. 1A, lanes 7–14, and Fig. 1B).

PKR–PKR protein interactions occur *in vivo* and are not dependent upon RNA-binding activity

The yeast two hybrid system (Fields and Song, 1989; Durfee *et al.,* 1993) was used to test the ability of PKR to interact with itself *in vivo.* Plasmids were constructed that encoded PKR proteins fused at their N-termini to either the DNA-binding domain (bd) or the transcription activation domain (ad) of the yeast Gal4 protein. The various PKR proteins examined in this way included the full-length single (K296R) and double (K64E/K296R) point mutants, and the truncated N-terminal RNA binding and C-terminal catalytic domains of the human PKR (Table 1). Because of the growth suppression activity of PKR(wt) expressed in yeast (Chong *et al.,* 1992), the full-length PKR(K296R) mutant lacking enzymic activity was used as the reference construction. Plasmids encoding these hybrid PKR–GAL4 proteins were coexpressed in the yeast Y190 host possessing two easily detectable reporter genes, histidine (*HIS*3) and β-galactosidase (*lac*Z), under the control of GAL4. Leucine and tryptophan auxotrophic markers were used to select for yeast that had been transformed with the activation or DNA-binding domain plasmids. The dual-detection system was subsequently used to screen for yeast that had been transformed with two plasmids encoding PKR–PKR interacting proteins; *HIS*3 gene expression was detected by plating transformants on media lacking histidine, and *lac*Z gene expression was detected by filter lift assay.

Pairwise combinations of GAL4bd and GAL4ad PKR protein fusions were examined and the results are summarized in Table 1. In the first series of experiments, the interaction of full-length PKR(1–551)K296R was examined. Cells expressing both GAL-PKR fusions as full-length PKR proteins, that is GAL4bdPKR(1–551)K296R and GAL4adPKR(1–551)K296R, grew in the absence of histidine and synthesized β-galactosidase. By contrast, cells expressing only one of the GAL4 domains as a full-length PKR(1–551)K296R fusion protein and the other GAL4 domain alone without fused PKR did not grow on media lacking histidine and did not express β-galactosidase (Fig. 2). In the next series of experiments, truncated PKR proteins were examined. Cells expressing GAL4bdPKR(1–551)K296R and the N-terminal RNA-binding domain of PKR [GAL4adPKR(1–280)wt], or GAL4adPKR(1–551)K296R and the C-terminal catalytic domain of PKR [GAL4bdPKR(281–551)K296R], did not grow in the absence of histidine and did not induce β-galactosidase. However, results obtained with the N-terminal RNA-binding domain of PKR [GAL4adPKR(1–280)wt] were somewhat variable between independent analyses, suggesting that this domain alone may possess a weak intermolecular PKR interaction activity. Finally, GAL4–PKR fusion proteins containing the K64E point mutation were tested. The double mutant PKR(K64E/K296R) did not bind double-stranded RNA, whereas the single mutant PKR(K296R) retained full RNA binding activity (McCormack and Samuel, 1995). Cells expressing one of the GAL4 domains as the full-length PKR(1–551)K64E/296R fusion protein and the other as a GAL4 domain fused to PKR(1–551)K296R grew on media lacking histidine and expressed β-galactosidase. Furthermore, cells expressing both GAL–PKR fusions as full-length double point mutant PKR(K64E/K296R) proteins, that is GAL4bdPKR(1–551)K64E/K296R and GAL4adPKR(1–551)K64E/K296R, also grew in the absence of histidine and synthesized β-galactosidase (Table 1; Fig. 2). Independent analyses of β-galactosidase expression in liquid culture using cell-free extracts prepared from cotransformed Y190 were consistent with the results of the filter lift assays using whole cells (data not shown).

The *S. cerevisiae* SNF1 and SNF4 GAL4 fusion proteins were used as positive controls (Fields and Song, 1989); when jointly expressed in transformed Y190 cells, they induced β-galactosidase and the transformants grew in media lacking histidine. A number of negative controls were also examined. The full-length PKR(1–551)K296R mutant fused to the GAL4ad did not detectably interact with RAS, RAS2, p53, or BCL2 fusions with the GAL4bd (Table 1).

Both the phosphotransfer-negative K296R mutant and the RNA-binding-negative K296R/K64E double mutant of PKR enhance reovirus reporter gene expression in cotransfected monkey cells

The reovirus s1 mRNA is inefficiently translated relative to the reovirus s4 mRNA, both in virus-infected and vector transfected cells (Zweerink and Joklik, 1970; Munemitsu and Samuel, 1988). The inefficiently translated reovirus s1 mRNA is a potent activator of the PKR kinase

TABLE 1

Homomeric Interaction of PKR Detected by the Two-Hybrid System

Transformant		Growth		
DNA binding domain hybrid	Activation domain hybrid	trp⁻, leu⁻ medium	trp⁻, leu⁻, his⁻ medium	Colony color
GAL4bd	GAL4ad	+	−	White
GAL4bd	GAL4adPKR(1-551)K296R	+	−	White
GAL4bdPKR(1-551)K296R	GAL4ad	+	−	White
GAL4bdPKR(1-551)K296R	GAL4adPKR(1-551)K296R	+	+	Blue
GAL4bdPKR(1-551)K296R	GAL4adPKR(1-280)wt	+	−	White
GAL4bdPKR(281-551)K296R	GAL4adPKR(1-551)K296R	+	−	White
GAL4bdPKR(1-551)K296R	GAL4adPKR(1-551)K64E/K296R	+	+	Blue
GAL4bdPKR(1-551)K64E/K296R	GAL4ad	+	−	White
GAL4bdPKR(1-551)K64E/K296R	GAL4adPKR(1-551)K296R	+	+	Blue
GAL4bdPKR(1-551)K64E/K296R	GAL4adPKR(1-551)K64E/K296R	+	+	Blue
GAL4bdPKR(1-551)K64E/K296R	GAL4adPKR(1-280)wt	+	−	White
GAL4bdRAS	GAL4adPKR(1-551)K296R	+	−	White
GAL4bdRAS2	GAL4adPKR(1-551)K296R	+	−	White
GAL4bdP53	GAL4adPKR(1-551)K296R	+	−	White
GAL4bdBCL2	GAL4adPKR(1-551)K296R	+	−	White
GAL4bdSNF1	GAL4adSNF4	+	+	Blue

Note. Y-190 cells were cotransformed with expression vectors encoding various GAL 4 DNA binding domain (GAL4bd) and GAL4 transcription activation domain (GAL4ad) fusion proteins as indicated, Wild-type and mutant forms of the RNA-dependent protein kinase (PKR) cDNA are as previously described (Thomis and Samuel, 1992; McCormack *et al.,* 1994); the RAS, RAS2, p53, and BCL2 (Van Aelst *et al.,* 1993) fusions with the GAL4bd were generously provided by Dr. R. Ballester (Univ. of Calif., Santa Barbara). For the growth experiments, aliquots of the same transformation mixture were plated on either synthetic dextrose plates lacking tryptophan and leucine or plates lacking tryptophan, leucine, and histidine but containing 25 m*M* 3-aminotriazole; plus (+) signs indicate growth of the transformed yeast colonies on the respective plates. Similar numbers of transformants from the same transformation mixture were obtained on plates lacking trp, leu, and his, and on plates lacking trp and leu only. β-galactosidase (X-Gal) activity was determined by the colony nitrocellulose filter lift method. Blue colony color indicates transcriptional activation of the *lac*Z reporter under the control of GAL4 binding site, which requires PKR–PKR interaction to bring the GAL4 activation and DNA binding domains of the GAL4–PKR fusion proteins together. White colony color indicates a lack of interaction between GAL4–PKR fusion proteins sufficient to activate transcription.

relative to the efficiently translated s4 mRNA which is a poor activator of PKR (Bischoff and Samuel, 1989). Cotransfection with full-length PKR(K296R) (Henry *et al.,* 1994) or treatment with 2-aminopurine (Samuel and Brody, 1990) increases the translational efficiency of reovirus s1 mRNA in S1(wt)-transfected cells about fivefold without significantly affecting the translation of s4 mRNA. A region of s1 mRNA sufficient for PKR activation has been mapped by deletion analysis to nt 416 to 576, and the chimeric hybrid S1/S4 gene that contains this PKR activator region within the 5′-half of the S1 gene fused in-frame with the 3′-half of the S4 gene is expressed inefficiently like that of the S1(wt) gene (Bischoff and Samuel, 1989; Henry *et al.,* 1994). To examine whether RNA binding activity of PKR was required for the transdominant phenotype that is characteristic of catalytic-deficient mutant PKR proteins in transfected cells (Koromilas *et al.,* 1992; Meurs *et al.,* 1993; Henry *et al.,* 1994), the expression of reovirus S class genes was analyzed in cells cotransfected with either the PKR(K296R/K64E) double mutant or the PKR(K296R) single mutant.

As shown in Fig. 3, the expressions of reovirus σ1(wt) protein (lane a) and σ1/σ3 hybrid protein (lane g) were much less efficient than that of σ3(wt) protein (lane d) in the absence of coexpression with mutant PKR when measured in [^{35}S]methionine pulse-labeled cells. However, over-expression of the catalytic deficient PKR(K296R) mutant in cotransfected cells increased the synthesis of S1-encoded σ1(wt) and S1/S4-encoded σ1/σ3 proteins but not S4-encoded σ3(wt) protein. This transdominant effect of PKR(K296R) on reovirus protein synthesis was not dependent upon RNA binding activity of the PKR protein. The K296R/K64E double mutant which lacks RNA binding activity (lanes c and i) increased the synthesis of σ1 (lanes b and c) and σ1/σ3 (lanes h and i) to comparable levels as the K296R single mutant (lanes b and h). As controls, no σ proteins were immunoprecipitated with reovirus antiserum from extracts prepared from cells not transfected with a reovirus expression construct, but transfected with either PKR(K296R) or PKR(K296R/K64E) alone (lanes j and k). When corrected for differences in methionine content, in the absence of PKR cotransfection the efficiency of synthesis of σ3(wt) protein in S4-transfected cells was about five times greater than that of the σ1(wt) or σ1/σ3 hybrid protein which were comparably expressed. These differences in σ-class protein synthesis were largely due to differences in their rates of synthesis rather than turnover; the levels of stable S-class-specific mRNA expressed from the three vectors were comparable and unaffected by PKR cotransfection, and the $t_{1/2}$ of the encoded

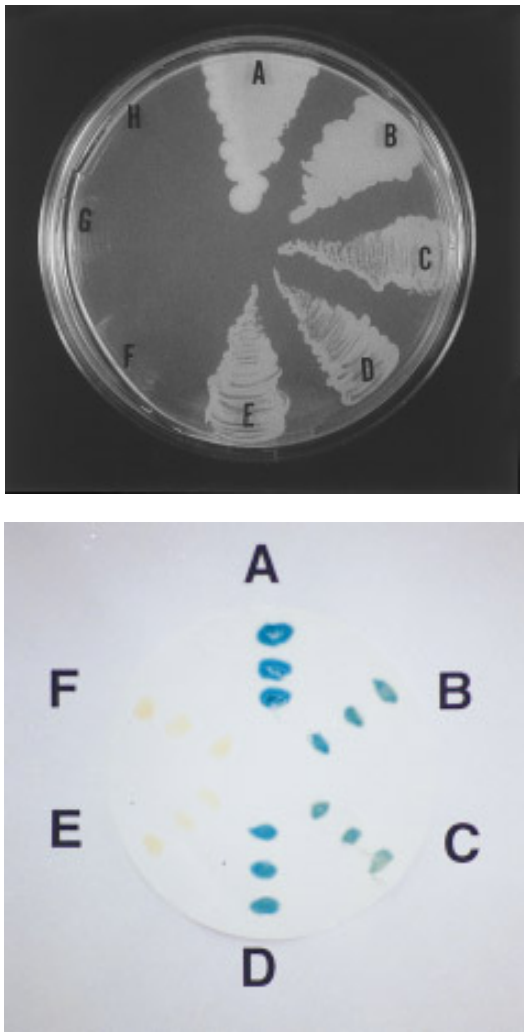

FIG. 2. Growth and β-galactosidase analysis of yeast strains coexpressing PKR proteins as Gal4 fusions. Plasmids carrying the indicated PKR-Gal4 fusion constructions, or control plasmids, were introduced into yeast strain Y-190 and β-galactosidase activity was determined by the nitrocellulose lift method as described for Table 1. (Top) Growth on trp$^-$ leu$^-$ his$^-$ plates. A, GAL4bdSNF1 and GAL4adSNF4; B, GAL4bdPKR(1–551)K296R and GAL4adPKR(1–551)K296R; C, GAL4bdPKR(1–551)K296R and GAL4adPKR(1–551)K64E/K296R; D, GAL4bdPKR(1–551)K64E/K296R and GAL4adPKR(1–551)K296R; E, GAL4bdPKR(1–551)K64E/K296R and GAL4adPKR(1–551)K64E/K296R; F, GAL4bdPKR(1–551)K296R and GAL4adPKR(1–280)wt; G, GAL4bdPKR(1–551)K296R and GAL4ad; and H, GAL4bd and GAL4ad. (Bottom) β-Galactosidase activity was determined by the nitrocellulose lift method. A, GAL4bdSNF1 and GAL4adSNF4; B, GAL4bdPKR(1–551)K296R and GAL4adPKR(1–551)K296R; C, GAL4bdPKR(1–551)K296R and GAL4adPKR(1–551)K64E/K296R; D, GAL4bdPKR(1–551)K64E/K296R and GAL4adPKR(1–551)K64E/K296R; E, GAL4bdPKR(1–551)K296R and GAL4adPKR(1–280)wt; and F, GAL4bdPKR(1–551)K296R and GAL4ad.

proteins estimated from pulse–chase analyses were about 6.4, 3.8, and 3.1 hr for the σ1(wt), σ3(wt), and σ1/σ3 proteins, all much longer than the 30-min pulse used for measurement of protein production (data not shown; Henry *et al.,* 1994).

DISCUSSION

Two important points emerge from the results of the biochemical and genetic analyses reported herein. First, the interferon-inducible protein kinase forms intermolecular PKR–PKR complexes, both *in vivo* and *in vitro.* Second, RNA binding activity of PKR is neither necessary nor sufficient for PKR–PKR protein complex formation *in vivo.* These findings may be related to several reports in the literature concerning the dominant negative phenotypes observed in transfected cells expressing PKR mutants that lack kinase activity. The demonstration that PKR associates with itself, both *in vivo* within intact cells in culture and also as a purified tagged protein *in vitro,* provides an explanation for the dominant negative property of PKR(K296R) (Barber *et al.,* 1993, 1995; Chong *et al.,* 1992; Henry *et al.,* 1994; Koromilas *et al.,* 1992; Meurs *et al.,* 1993; and Romano *et al.,* 1995).

The results that we obtained using two complementary *in vivo* assay systems, the yeast two-hybrid assay and the reovirus S1 reporter assay in monkey COS cells, clearly establish that RNA-binding mutants can act as dominant negative inhibitors of PKR function. The structure–function data from the two-hybrid analysis indicates that neither the N-terminal half of PKR corresponding to the RNA-binding R domain nor the C-terminal half of PKR corresponding to the catalytic C domain are sufficient to mediate PKR–PKR protein interaction. Our findings that RNA binding is neither necessary nor sufficient for PKR–PKR intermolecular protein interaction as measured with the yeast two-hybrid system are in agreement with the report that mutants of PKR lacking the N-terminal RNA binding motif R_I, which is of primary importance for RNA binding activity (McCormack *et al.,* 1994), can act as transdominant inhibitors and induce malignant transformation (Barber *et al.,* 1995). Patel *et al.* (1995) also recently demonstrated PKR–PKR protein interactions with the mammalian COS cell system and by affinity chromatography; like our results, they found that a PKR mutant that lacked dsRNA binding activity could still exhibit PKR–PKR interaction activity. Together, the results reported herein and those of Barber *et al.* (1995), Romano *et al.* (1995), and Patel *et al.* (1995) strongly argue that the dominant-negative characteristics of PKR catalytic-deficient full-length mutants such as PKR(K296R) derive from the formation of inactive PKR–PKR heterodimers rather than sequestering activator RNAs.

Structure–function analyses using the two-hybrid system suggest that the region of PKR corresponding to the hinge region between the R and C domains may be required for efficient intermolecular PKR association, and that the appropriate conformation of this region is modulated by the N-terminus of PKR. This would provide an explanation for the properties reported for the deletion mutant PKR(98–551), which was not a transdominant inhibitor (Romano *et al.,* 1995), even though various point mutants lacking RNA-binding activity do function as

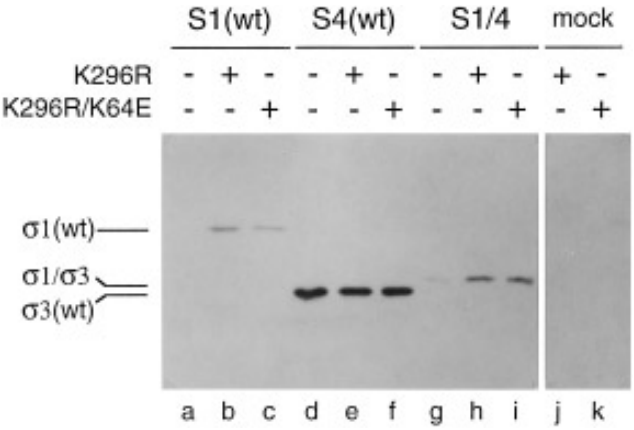

FIG. 3. Effect on the expression of wild-type and chimeric reovirus genes of cotransfection with either full-length K296R single or K296R/K64E double mutant PKR protein. COS cells were transfected with the indicated reovirus S-class expression vector, either alone (−) or with the full-length catalytic deficient PKR mutant K296R (+, lanes b, e, h, and j) or the PKR double mutant K296R/K64E which lacks RNA binding activity (+, lanes c, f, i, and k). Lanes a–c, cells transfected with wild-type S1 vector encoding σ1 protein; lanes d–f, cells transfected with wild-type S4 vector encoding σ3 protein; lanes g–i, cells transfected with the mutant S1/S4 vector encoding the hybrid σ1/σ3 protein; lanes k and l, cells mock-transfected with vector alone. Cells were pulse-labeled with [^{35}S]methionine at 48 hr after transfection, extracts were prepared, and the reovirus proteins were immunoprecipitated and analyzed by SDS–PAGE (10% polyacrylamide gel) and autoradiography. The positions of the reovirus proteins are indicated to the left of the autoradiogram.

transdominant inhibitors (herein; Barber *et al.,* 1995). Although it has been reported that PKR(98–551) retains activity in a reporter assay (Lee *et al.,* 1994), other investigators have found that such PKR deletion mutants lacked both enzyme and growth suppression activities (Barber *et al.,* 1995; Romano *et al.,* 1995). However, the absence of a detectable activity in an assay involving a deletion mutant could result if the deleted region of the protein specified the actual site of a function, or alternatively, if the deleted region simply caused a conformation change in the protein that abrogated function without the deleted region per se being significant.

The ability of PKR mutants to reverse the growth-suppressive phenotype of PKR(wt) and to complement GCN2 in yeast revealed that the N-terminal RNA-binding motif was necessary, and that mutants of the two RNA binding R motifs functionally complemented each other in yeast (Romano *et al.,* 1995). The latter result suggests that dimerization may be necessary for activation. Indeed, in mouse cells, PKR isolated from the cytosolic S100 supernatant fraction appears to be a dimeric protein of 140- to 160-kDa size (Langland and Jacobs, 1992), whereas PKR from the ribosomal salt-wash fraction is reported to be a dephosphorylated monomer of about 62 to 66-kDa size (Langland and Jacobs, 1992; Berry *et al.,* 1985). Our demonstration using the yeast two-hybrid system that PKR–PKR interactions occur *in vivo* (Table 1; Fig. 2), together with the reports that catalytically inactive PKR(K296R) will reverse the growth suppressive phenotype of PKR(wt) in yeast (Chong *et al.,* 1992; Romano *et al.,* 1995) and mediate transformation in the NIH 3T3 cell assay (Barber *et al.,* 1995), are consistent with the notion that the active form of PKR may be a dimer and that heterodimers lack catalytic activity.

Our finding that the synthesis of reovirus σ1 protein was enhanced in monkey COS cells by coexpression of the catalytically inactive PKR(K296R) mutant is in full agreement with the previously reported observations of Henry *et al.* (1994) obtained with reovirus reporter proteins and of Barber *et al.* (1993) obtained with a secreted phosphatase as the reporter protein. The enhancement of the reporter protein expression observed in transfected cells by coexpression of the K296R mutant of PKR was consistent with a mechanism involving competition between endogenous PKR(wt) and exogenously introduced PKR(K296R) for the binding of activator RNAs. The possibility that PKR(K296R) simply sequestered activator RNAs gained support from results with heterologous RNA binding proteins. Both truncated PKR(1–243) possessing the N-terminal RNA binding domain but not the C-terminal catalytic domains of PKR, and the vaccinia virus RNA-binding protein E3L, increased reovirus S1 expression *in vivo* in cotransfected cells similar to the level observed with full-length PKR(K296R) (Henry *et al.,* 1994). However, the formation of inactive heterodimers between transfected PKR(K296R) and endogenous PKR(wt) would also provide a second mechanism for the observed dominant negative behavior of PKR. Our finding that the PKR double mutant K296R/K64E lacking both catalytic and RNA binding activity also enhanced the expression of the reovirus S1 but not S4 genes in transfected cells establishes that competition for activator RNAs cannot be the sole mechanism for the dominant negative behavior of the K296R mutant. The results are consistent with the notion that inactive heterodimers form between endogenous PKR(wt) and either PKR(K296R) or PKR(K296R/K64E) and are in full agreement with our results from the two-hybrid analyses which establish that RNA-binding activity is neither sufficient nor required for PKR–PKR complex formation in yeast.

The observation that purified His-PKR(K296R) antagonized the catalytic activity of purified PKR(wt) kinase *in vitro* is in agreement with the results of Sharp *et al.* (1993) who found that PKR(K296R) purified from baculovirus-infected cells rescued the endogenous protein synthesis activity of a rabbit reticulocyte cell-free system from inhibition by low concentrations of double-stranded RNA. We found that both the autophosphorylation of PKR(wt) enzyme and the intermolecular phosphorylation of His-PKR(K296) were inhibited in the presence of a low concentration of dsRNA by addition of excess His-PKR(K296R). Because the effects of PKR(K296R) can be reversed by addition of higher concentrations of activator dsRNA to the phosphorylation reaction mixture (Sharp *et al.,* 1993), in this case one of the mechanisms of inhibition

likely involves simple competition between the wild-type and mutant PKR proteins for the activator RNA. Indeed, reports that heterologous dsRNA binding proteins such as the reovirus σ3 protein (Imani *et al.,* 1988) and the vaccinia virus E3L protein (Chong *et al.,* 1992) can prevent PKR activation *in vitro* by sequestering the activator RNA support this notion. However, the fact that purified PKR(wt) catalyzes the RNA-dependent phosphorylation of His-PKR(K296R) establishes that a heteromeric complex between the wild-type and mutant PKR proteins can form *in vitro,* at least in the context of an enzyme–substrate interaction. Interestingly, phosphopeptide analysis has revealed that the sites of intermolecular autophosphorylation in His-PKR(K296R) are very similar, if not identical, to the sites that are autophosphorylated in PKR(wt) (Thomis and Samuel, 1995).

In summary, the combined results of the biochemical and genetic analyses of the dominant negative behavior of mutant PKR proteins clearly reveal that two different mechanisms may be operative. One mechanism is independent of the RNA binding activity of PKR, as established by the ability of RNA-binding-deficient PKR mutants to mediate the dominant-negative phenotype. By contrast, the other mechanism has as its basis the RNA binding activity of PKR as established by the ability of heterologous RNA binding proteins to substitute for the catalytically inactive PKR in mediating the effect. The demonstration that PKR–PKR interactions occur *in vivo,* and that the RNA binding activity of PKR is neither necessary nor sufficient for PKR intermolecular association, is relevant to the former mechanism. It is now of upmost importance to establish in biochemical and biophysical terms the nature of the PKR–PKR intermolecular protein interactions and then to attempt to define the roles that established effectors of kinase function, including various viral and cellular proteins and RNAs (Samuel, 1993; Mathews, 1993; Katze, 1995), possibly play in modulating PKR–PKR protein interactions and PKR kinase functions.

ACKNOWLEDGMENTS

This work was supported in part by Research Grant AI-20611 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and was presented in part at the Annual Meeting of the International Society for Interferon and Cytokine Research, Budapest, October, 1994.

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